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## **Effects of Humic Acid Application on Growth and Biomass Production of *Azolla Pinnata* in Symbiosis with *Anabaena Azollae* Under Semi-Arid Conditions**

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### **Abstract**

*Azolla pinnata* is a fast-growing aquatic fern known for its unique ability to form a symbiotic relationship with the nitrogen-fixing cyanobacterium *Anabaena azollae*. This association allows it to capture atmospheric nitrogen, which promotes the development of biomass rich in protein and nutrients. This symbiotic relationship represents an interesting biological model for semi-arid regions. Our study aims to evaluate the effect of humic acid, alone or combined with manure, on the growth of *A. pinnata* associated with *A. azollae* under a semi-arid climate. The materials used in the experiment were plastic containers filled with soil, water (distilled or well water), and fresh *A. pinnata* associated with *A. azollae*, subjected to three concentrations of humic acid (2, 4, and 6 g.L<sup>-1</sup>), with or without manure, over a 7-day period in a semi-arid climate. The results showed that the

T0/C2 treatment (+ 4 g.L<sup>-1</sup> humic acid without manure) induced strong growth (+48.6% biomass), accompanied by an increase in protein concentrations (21.54 mg. g<sup>-1</sup> FM) and carbohydrate concentrations (96.5 mg. g<sup>-1</sup> FM), reflecting improved photosynthesis and symbiosis under semi-arid conditions. In contrast, the T+/C3 treatment (manure + high humic acid) reduced growth, likely due to an excess of organic matter disrupting osmotic balance. The positive correlations observed were for protein ( $r = 0.904$ ), carbohydrates ( $r = 0.779$ ), chlorophyll content ( $r = 0.724$ ) and negative correlation are observed for a stress marker proline ( $r = -0.649$ ). In conclusion, humic acid, when used in moderate doses, stimulates the growth of *A. pinnata* in association with *A. azollae* under unfavorable conditions.

**Keywords:** *Azolla Pinnata*, *Anabaena Azollae*, Humic Acid, Growth, Semi-Arid Conditions, Symbiosis

### **Introduction**

*Azolla* has played an important role in agriculture throughout history. Since ancient times, it has been recognized as a valuable ally in southern China and northern Vietnam. Used as a biofertilizer and green manure for rice cultivation, its effectiveness stems from its remarkable ability to fix atmospheric nitrogen. Furthermore, *Azolla* was also used as feed for farmyard animals [1]. Aquatic plants such as *Azolla pinnata* characterized by a high protein content, which makes it increasingly valuable in the agricultural sector. It is one of the few alternative forage crops capable of providing usable protein, particularly in systems where traditional resources are limited. *Azolla* also has the advantage of rapid growth [2]. Recent research has highlighted the potential of *Azolla pinnata* as a sustainable supplementary feed for various types of farm animals. With a protein concentration ranging from 25% to 30% and a high-quality amino acid profile that stimulates growth and productivity in these animals, while reducing their dependence on traditional protein sources such as soybean meal [3]. Moreover, symbiosis between the aquatic fern *Azolla pinnata* and the Blue-Green Algae *Anabaena azollae* is a remarkable biological model of mutualistic association, capable of naturally fixing atmospheric nitrogen. This symbiotic interaction is attracting growing interest in sustainable agriculture, particularly in tropical rice-growing systems [4]. Humic acid is one of the humic substances, along with fulvic acids and humins. It is formed during the decomposition of organic matter, primarily plant matter, and is characterized by its complex chemical structure, which is rich in functional groups such as carboxyl and phenolate groups [5]. Humic acid plays a central role in soil biological processes, influencing nutrient availability and soil structure [6]. Humic acid helps plants better

withstand abiotic stresses such as drought, extreme temperatures, or excessive salt levels [7]. In addition, humic acids can protect lipids from peroxidation, one of the most harmful effects of reactive oxygen species (ROS) on cells during stressful periods [8].

### Materials and Methods

The experiment was conducted in Laboratory of AgroBiotech-URL-CNRST-05. Experiment lasted 7 days in a semi-arid climate. 15 g of soil and 5 g of fresh *Azolla* in symbiosis with *A. azollae* were used in each container for the treatments. Each treatment was tested with 3 concentrations of humic acid (2, 4, and 6 g.L<sup>-1</sup>) and three replicates per concentration:

Treatment 1 (T1): Soil + humic acid solution prepared with distilled water, without manure.

Treatment 2 (T2): Soil + humic acid solution prepared with well water, without manure.

Treatment 3 (T3): Soil + manure + humic acid solution prepared with well water.

Three control treatments were also established:

Negative control (T-): Soil + distilled water + *Azolla*, without humic acid or manure.

Positive control (T0): Soil + well water + *Azolla*, without humic acid.

Positive control (T+): Soil + manure + well water + *Azolla*, without humic acid.

### Measurement of Agro-Physiological Parameters

#### Fresh biomass

To evaluate the effect of humic acid-based treatments on the growth of *Azolla pinnata*, the fresh matter (FM) of the biomass was measured using an electronic scale at the beginning and end of the experiment. The percentage increase in mass was calculated using the following formula: Variation in fresh biomass (%) =  $((W_2 - W_1) / W_1) \times 100$ , Where:  $W_1$ : initial fresh weight (g) and  $W_2$ : final fresh weight (g).

#### Determination of chlorophyll content

Chlorophyll (Chl) concentration was determined using the method described by Arnon [9]. In a mortar, Chl was extracted with 5 mL of acetone (80%, v/v), using 200 mg of fresh leaf. After 7 min centrifugation (5000 × g), the chlorophyll concentration was determined in the supernatants using a spectrophotometer at OD (optical densities) of 663 and 645 nm respectively and evaluated using the formula:  $\text{Chl (mg. g}^{-1} \text{ FM)} = (8.02 \times \text{OD}_{663} + 20.2 \times \text{OD}_{645})$ .

#### Determination of soluble sugars content

A 100-mg sample of seeds is ground in 0.5 mL of 95% ethanol, followed by three rinses with 70% ethanol; the ground sample is then centrifuged at 5000 g for 10 minutes. Next, 100 µl of the supernatant is placed in a tube, to which 3 ml of an anthrone solution (prepared by dissolving 1.5 g of anthrone in 1 L of 72% sulfuric acid) is added. The tubes are brought to a boil in a water bath for 10 minutes. The optical density is measured using a spectrophotometer at 625 nm. The concentration of soluble sugars is determined using a calibration curve prepared with glucose solutions [10].

### Determination of protein content

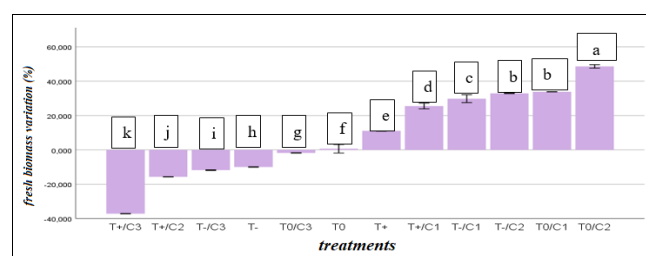
Assay is performed according to the method described by Bradford [11]. Proteins are extracted by grinding 50 mg of seeds in 2 mL of phosphate buffer (pH 6.8). The samples are then centrifuged at 12,500 g for 20 minutes. A 100 µL aliquot of the supernatant is placed in a tube containing 100 µL of distilled water and 2 mL of Bradford reagent. The tubes are shaken and allowed to stand for 5 min at a temperature of 27°C. Absorbance is measured by spectrophotometer at a wavelength of 595 nm. Protein concentrations are determined using a calibration series of BSA (Bovine Serum Albumin) solutions.

### Determination of proline content

The proline assay was performed according to the method of Bates [12], based on the reaction of proline with ninhydrin reagent in an organic medium. For each sample, 200 mg of fresh *Azolla* material was homogenized in 10 mL of 70% ethanol using a mortar. The mixture was then incubated in a water bath at 100 °C for 30 minutes to facilitate proline extraction. After cooling, the samples were centrifuged at 1300 rpm for 10 minutes. A 500 µl volume of the supernatant was removed and placed in a test tube, then mixed with 2 ml of ninhydrin reagent. The tubes were incubated in a water bath at 95 °C for 10 minutes to allow the color to develop. After cooling, 3 mL of toluene was added to each tube, then gently shaken for 15 to 20 seconds. After decanting, the upper phase (colored toluene layer) containing the proline–ninhydrin complex was recovered. Absorbance was measured using a spectrophotometer at a wavelength of 520 nm. The proline concentration was determined from a calibration curve prepared using known concentrations of standard proline.

### Results and Discussion

**Fresh biomass** was measured at the end of the experiment, and the weight gain index was calculated relative to the initial biomass. The following figure shows the average values obtained for each treatment.



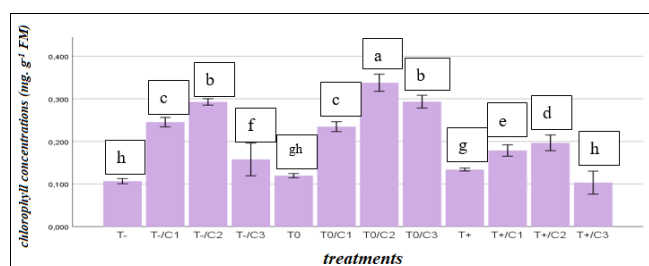
**Fig 1:** Fresh biomass variation (%) of *A. pinnata* in symbiosis with *A. azollae*, depending on the different treatments; bars represent means ± standard errors, with letters indicating significant differences at the 0.05 probability level according to the Student-Newman-Keuls (SNK) test

Results showed that humic acid had a significant effect ( $p < 0.05$ , Table 1) on the fresh biomass of *Azolla* combined with *A. azollae*. Indeed, the negative control (T-) showed a significant decrease in fresh matter (-37.15), followed by the positive control T0 with slight growth (0.69%), while the T+ control (manure + well water) significantly improved growth (11.11%) (Fig 1). Meanwhile, treatments with humic

acid significantly improved fresh biomass, as seen in T0/C2 (48.6%), T0/C1 (33.8%), and T+/C1 (32.9%). The study of the three concentrations (2, 4, and 6 g.L<sup>-1</sup>) showed that T+/C1 (2 g.L<sup>-1</sup>) yielded high growth (32.9%), but this growth dropped sharply with higher concentrations of humic acid, such as T+/C2 (4 g.L<sup>-1</sup>, -15.7%) and T+/C3 (6 g.L<sup>-1</sup>, -37.15%). This suggests that high doses of humic acid, such as 6 g.L<sup>-1</sup>, may be phytotoxic or cause osmotic stress. The concentration of 4 g.L<sup>-1</sup> (C2) is the optimal concentration, particularly when applied without manure (T0). The high temperatures recorded (up to 38.4 °C) exceeded the optimal range recommended for *Azolla* cultivation (20–30 °C) according to [13], which may have induced heat stress, partially reducing overall growth. Furthermore, relative humidity (48–51.5%) was significantly lower than the optimal range (80–90%) according to [14], which may also have limited photosynthesis and nitrogen fixation. According to [15], the growth of *Azolla pinnata* can reach up to 194% by the 15th day under optimal conditions.

### Results of chlorophyll content

The treatments had a significant effect on the average chlorophyll concentrations observed in the various trials compared to the control. The observed values are concordant with those reported in the literature [16], which demonstrated the negative effect of stress on chlorophyll content. On the other hand, the positive effects of humic acid (HA) have been observed; in fact, the results showed a significant increase ( $p < 0.001$ , Table 1) in total chlorophyll in the T-/C2 and T0/C2 treatments, with concentrations of 0.293 and 0.337 mg. g<sup>-1</sup> FM, respectively, which can be attributed to stimulation of the *Azolla*–*Anabaena* symbiosis by humic acid, notably through increased membrane permeability, facilitating nutrient transfer [17], and Several studies have shown that the application of HA improved the availability of P, K, Fe, and Mg in the soil, as well as the content of amino acids and the concentrations of soluble sugars and betaine; in particular, it enhanced photosynthesis, carbon fixation, and hormonal and osmotic metabolism in the leaves [8, 18].

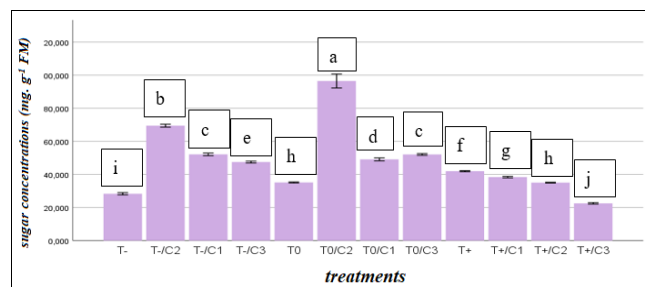


**Fig 2:** Average chlorophyll concentrations (mg. g<sup>-1</sup> FM) as a function of the different treatments applied to a culture of *A. pinnata* in symbiosis with *A. azollae*. Bars represent means  $\pm$  standard errors, with letters indicating significant differences at the 0.05 probability level according to the Student-Newman-Keuls (SNK) test

### Results of soluble sugars content

Treatments T0/C2 (well water with 4 g.L<sup>-1</sup> humic acid) and T-/C2 (distilled water with 4 g.L<sup>-1</sup> HA) induce the highest values for all three parameters, while T+/C3 (manure with 6 g.L<sup>-1</sup> HA) showed the lowest value, suggesting an inhibitory effect at this concentration (Fig 3). Indeed, results showed a significant increase of sugar content ( $p < 0.001$ ) in

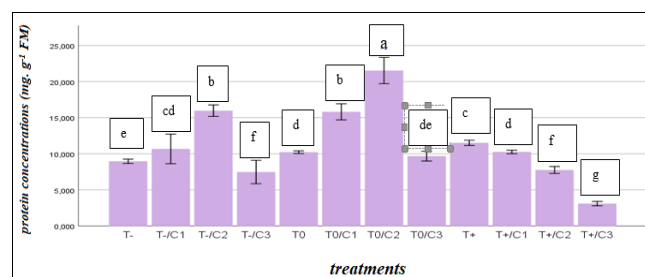
the T0/C2 and T+/C2 treatments, with concentrations of 96.490 and 69.487 mg. g<sup>-1</sup> FM, respectively. Total sugars represent the primary production resulting from photosynthesis, which supplies cyanobacteria with reduced carbon, among other things, which is essential for nitrogen fixation. The addition of humic acid intensified metabolic exchange in the symbiosis [19, 20]. The results are consistent with several studies that have demonstrated the positive effects of humic substances on plant growth, in part by acting on the plasma membrane to promote the absorption of certain nutrients [17].



**Fig 3:** Average sugar concentrations (mg. g<sup>-1</sup> FM) as a function of the different treatments applied to a culture of *A. pinnata* in symbiosis with *A. azollae*. Bars represent means  $\pm$  standard errors, with letters indicating significant differences at the 0.05 probability level according to the Student-Newman-Keuls (SNK) test

### Results of protein content

The results showed that protein concentrations were significantly ( $p < 0.001$ ) affected by the humic acid treatments. In fact, a significant increase in protein content was observed in the T0/C2 and T+/C2 treatments (21.5 and 16 mg. g<sup>-1</sup> FM, respectively; Fig 4). Humic acid stimulates symbiotic nitrogen fixation; this phenomenon can be explained by a direct influence of humic acid-induced protein synthesis and improved solubilization of trace elements essential for nitrogen fixation, such as Fe and Mo. authors have also shown the positive effect of humic acid on biological nitrogen fixation, the stimulation of plant growth, and the symbiosis of host plants under abiotic stress [21, 22]. Humic acids (HA) play a key role in primary plant metabolism by intervening in carbon and nitrogen cycles and also have the ability to change the secondary metabolism of plants by influencing gene expression and thereby inducing the synthesis of compounds that help plants resist abiotic stress [22, 23]. Furthermore, the biochemical composition reported by [24], showed that *Azolla pinnata* is naturally rich in protein (up to 17.61%).



**Fig 4:** Average protein concentrations (mg. g<sup>-1</sup> FM) as a function of the different treatments applied to a culture of *A. pinnata* in symbiosis with *A. azollae*. Bars represent means  $\pm$  standard errors, with letters indicating significant differences at the 0.05 probability level according to the Student-Newman-Keuls (SNK) test

## Conclusion

The results clearly showed that the T0/C2 treatment performed best under adverse conditions. Indeed, the highest values for chlorophyll and protein content under abiotic stress were recorded with this treatment, indicating active stimulation of photosynthesis and an intensification of the symbiosis between *Azolla* and *Anabaena*. Furthermore, the moderately enriched mineral environment of the well water, combined with the action of the humic biostimulant, appears to have optimized nutrient availability without causing organic overload. Additionally, humic acid has demonstrated its effectiveness as a sustainable method in crop production and has helped mitigate the negative effects of climate change.

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